ESCHERICHIA COLI HDEB IS AN ACID-STRESS CHAPERONE

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Abbreviations: DTT : dithiothreitol ; GAPDH : glyceraldehyde-3-phosphate dehydrogenase ; ADH : alcohol dehydrogenase ; ANS : 1-anilino-8-naphtalenesulfonate.

ABSTRACT

We have cloned, expressed and purified the *hdeB* gene product, which belongs to the *hdeAB* acid-stress operon. We extracted HdeB from bacteria by the osmotic shock procedure and purified it to homogeneity by ion exchange chromatography and hydroxyapatite chromatography. Its identity was confirmed by mass spectrometry analysis. HdeB has a molecular mass of 10 kDa in SDS-PAGE which matches its expected molecular weight. We purified the acid-stress chaperone HdeA in parallel in order to compare the two chaperones. The *hdeA* and *hdeB* mutants both display a reduced viability upon acid stress, and only the HdeA/HdeB expression plasmid can restore their viability close to the wild-type level, suggesting that both proteins are required for an optimal protection of the bacterial periplasm against acid stress. Periplasmic extracts from both mutants aggregate at acidic pH suggesting that HdeA and HdeB are required for protein solubilization. At pH 2, the aggregation of periplasmic extracts is prevented by the addition of HdeA, as previously reported, but only slightly reduced by HdeB. At pH 3, however, HdeB is more efficient than HdeA in preventing periplasmic protein aggregation. The solubilization of several model substrate proteins at acidic pH supports the hypothesis that HdeA plays a major role in protein solubilization at pH 2, and that both proteins are involved in protein solubilization at pH 3. Like HdeA, HdeB exposes hydrophobic surfaces at acidic pH, in accordance with the appearance of its chaperone properties at acidic pH. HdeB, like HdeA, dissociates from dimers at neutral pH into monomers at acidic pHs, but its dissociation is complete at pH 3, whereas that of HdeA is complete at a more acidic pH. Thus, we can conclude that *E. coli* possesses two acid stress chaperones that prevent periplasmic protein aggregation at acidic pH.

INTRODUCTION

In their natural habitats, bacteria are constantly under assault from a wide array of environmental stresses, including UV, heat, oxidative, osmotic and pH stresses (5). One of the most frequently encountered is acid stress (8). Enterobacteria, when travelling through the gastrointestinal tract encounter an extremely low pH; facultative intracellular pathogens tolerate episodes of low pH within macrophage phagolysosomes, and fermentative bacteria excrete acidic fermentation products that trigger an endogeneous acid stress (5, 17).

In response to acid stress, several mechanisms regulate the homeostasis of bacterial pH. Many bacteria, including *E. coli*, possess aminoacid (glutamate, arginine, or lysine) decarboxylase systems, each of which consists of a cytoplasmic decarboxylase which converts its substrate to a related amine (γ-aminobutyric acid, agmatine or cadaverine, respectively), and an antiporter which exchanges the imported amino acid for the cytoplasmic amine produced (5, 8, 12, 21, 24). These systems consume one cytoplasmic proton during amino acid decarboxylation, and one extracytoplasmic proton during protonation of the exported amine, thus leading to cytoplasmic and periplasmic alkalinization. In several bacteria, including fermentative bacteria such as *Lactococcus lactis*, the proton-translocating F_1F_0 ATPase can export protons as a consequence of ATP hydrolysis (26). Further protection against acid stress is obtained by decreasing the permeability of the inner and outer membranes to protons ; several inner and outer membrane proteins are overexpressed in response to acid stress, including the cyclopropane fatty acyl phospholipid synthase, OmpC, the LPS biosynthesis enzyme YfbF and the outer membrane lipoprotein Slp (26). Furthermore, bacteria can reverse their cytoplasmic membrane potential to an inside-positive potential that slows the influx of protons into the cell (8, 21). They can also reorient their metabolism towards pathways that decrease proton production or increase amine production with a consequent alkalinization (underexpression of catabolic sugar enzymes, such as those of the maltose regulon (27) decreases the production of organic acids, and overexpression of urease increases ammonia production in *Helicobacter pylori* (2, 7)). Moreover, several protective proteins may be induced upon acid stress, including the DnaK and GroEL chaperone machines (17) and several DNA repair enzymes (25).

The acid resistance mechanisms of the periplasm are not as well understood as those of the cytoplasm. Periplasmic proteins are probably more vulnerable than cytoplasmic proteins to acid stress, due to the relative permeability of the outer membrane porins to molecules smaller than 600 Da (15, 23). Recently, a periplasmic chaperone, HdeA, which supports acid resistance in *E. coli*, was discovered (9, 14). HdeA exhibits chaperone-like activity at strong acidic pH only (below pH 3), preventing the acid-induced aggregation of bacterial periplasmic extracts, and of several model substrate proteins such as alcohol dehydrogenase (ADH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the periplasmic ribose receptor (14).

In this study, we report the cloning, purification and characterization of HdeB as a novel acid-stress chaperone, and we show that, whereas HdeA is more efficient than HdeB in protein solubilization at pH 2, HdeB is more efficient than HdeA at pH 3. Thus, *E. coli* possesses two acid-stress chaperones, HdeA and HdeB, which are encoded by the acid-stress operon *hdeAB*, and prevent the aggregation of periplasmic proteins at acidic pH.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditons. The *E. coli* BL21 (DE3) strain (Novagen, USA) was used for the transformation of the new expression vector constructs containing the genes encoding for HdeB and HdeA, respectively. We generated the genes coding for HdeB and HdeA by amplifying the *hdeB* and *hdeA* genes from *E. coli* genomic DNA (from strain MG1655) by PCR using the forward primers (5'GGTGGTTGCTCTTCACATATGAATATTTCATCTCTC-3' for *hdeB*, and 5'GGTGGTTGCTCTTCACATATGAAAAAAGTATTAGGC-3' for *hdeA*) containing a *Nde1* site and the reverse primers (5'GGTGGTCTGGGATCCTCATTAATTCGGCAAGTCATT-3' for *hdeB*, and 5'GGTGGTCTGGAATCCTCATCATTACATATCTTTCTYTAAT-3' for *hdeA*) containing a *BamH1* site. The whole *hdeAB* operon was generated by PCR using the *hdeA* forward primer and the *hdeB* reverse primer. The PCR was done in a volume of 50 µl, in the presence of 0.5 µg of template DNA, 1 µg of forward and reverse primers and 5 units of Taq DNA polymerase (Applied Biosystems), for 35 cycles (1 min at 94°C, 2 min at 58°C, 2 min at 72°C°) followed by a final cycle (1 min at 94°C, 2 min at 58°C, 10 min at 72°C). The resulting products were digested with *Nde*1 and *BamH*1, ligated to the pET-21a (Novagen, USA) *Nde*1 and *BamH*1 backbone fragment, and transformed into strain BL21 (DE3). The sequences of the cloned genes were confirmed by DNA sequencing (not shown).

Construction of the *hdeA* and *hdeB* mutants. We achieved the one-step inactivation of the *hdeA and hdeB* genes using the phage λ recombination (Red) system (4). The strategy is analogous to the PCR-based gene deletion method in yeast, except that we used *E. coli* cells carrying an easily curable, low copy number plasmid pSC101 (replication temperature-sensitive derivative)-based plasmid pKD46 carrying the λ Red recombinase genes expressing the Red system. The basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene that is generated by PCR by using primers with 50-nt extensions homologous to the adjacent upstream or downstream flanking regions of the target gene and 20-nt 3' end for the amplification of the kanamycin *(kan)* resistance gene. This replacement was achieved by λ Red-mediated recombination in these flanking homologies (4). Strain BW25113 (*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* Δ*(araBAD)567* Δ(*rhaBAD)568 rph-1*) was used as the parent for construction of the *hdeA* and *hdeB* mutants. The construction of the *hdeA* and *hdeB* disruption mutants is part of the systematic construction of *E. coli* single gene deletion mutants (1). The amounts of HdeA and HdeB in the *hdeA*- and *hdeB*-deficient mutants, and in these mutants complemented with pBAD vectors (see below) were evaluated by 1D- or 2D-gel electrophoresis of osmotic shock fluids prepared from these strains.

Construction of pBAD vectors. Construction of pBAD vectors for *in vivo* expression of HdeA and HdeB in the *hdeA*- and *hdeB*-deficient strains was done as follows. The *hdeA* gene, the *hdeB* gene and the *hdeAB* operon were excised from the pET-21a-*hdeA,* pET-21a-*hdeB* and pET-21a-*hdeAB v*ectors using *Xba* I and *Hind* III, and ligated to the pBAD33 *Xba* I and *Hind* III backbone fragment (pBAD33 is a pACYC184-derived vector containing the P_{BAD} promoter of the *araBAD* operon and the gene encoding the regulator of this promoter, *araC*) (11). The pBAD vectors were induced with 0.05% arabinose.

Preparation of bacterial extracts, and purification of HdeA and HdeB. The HdeA and HdeB overproducing strains BL21 (DE3) pET-21a-*hdeA* and BL21 (DE3) pET-21a-*hdeB* were grown at 37°C in 1 liter of Luria-Bertani medium (20) supplemented with ampicillin (50 µg/ml) to an OD_{600} = 0.5. HdeA and HdeB overexpression was induced with 1 mM IPTG and growth was continued for 3 hours. Cells were harvested by centrifugation at 4°C. The cell pellets were resuspended in 10 ml of 30 mM Tris pH 8.0, 20 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and bacterial periplasmic extracts were prepared according to the osmotic shock procedure described in (13). HdeA and HdeB were each loaded onto a DEAE -Sephacel column equilibrated in 30 mM Tris pH 8.0, 1 mM DTT at 20°C, and eluted with a linear gradient of 0 – 0.5 M NaCl in the same buffer. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and quantified by the Bradford assay. Purified fractions of HdeB and HdeA were each loaded onto a hydroxyapatite column (Bio-Gel HTP from BioRad), equilibrated at 20°C in 30 mM Tris pH 7.5, 1 mM DTT and eluted with a linear gradient of 0 - 50 mM sodium

phosphate pH 7.5 in the same buffer. HdeA and HdeB were stored at -70°C. For experiments at acidic pH, HdeA and HdeB were equilibrated in distilled water by gel permeation on a Bio-Gel P10 column equilibrated in water (BioRad). We performed electrophoresis according to Laemmli, using 16% polyacrylamide gels (Bio-Rad) with Coomassie blue staining (16). All the experiments showing polyacrylamide gels (Figure 2-5) have been repeated at least three times, leading to similar results. We used NIH-Image 1.62 software (rsb.info.nih.gov/nih-image/Default/html) to quantify protein bands on polyacrylamide gels.

Mass spectrometric identification of HdeA and HdeB. Excised HdeA and HdeB gel bands were in-gel digested with mass spectrometry grade trypsin (Roche). Mass spectra were recorded in the positive ion reflection mode of a matrix -assisted laser desorption ionization-time of flight (MALDI-ToF) Voyager DE PRO (Applied Biosystems). The petide masses obtained were searched against the *E. coli* database (PIR, National Biomedical Research Foundation) with the Mascot engine available online (www.matrixscience.com).

Chaperone assays. The chaperone activity of HdeA and HdeB was assayed by incubating periplasmic extracts or model substrate proteins for 60 min at 25°C in the presence of HdeA and/or HdeB at acidic pH, and monitoring the appearance of the proteins in the 15,000 x g pellet or supernatant (protein were analyzed by SDS-PAGE) (14). Sulfate was used as the anion in order to achieve effective aggregation of substrate proteins at low pH values (10, 14).

ANS binding assay to HdeA and HdeB. Using a Kontron SFM 25 fluorescence spectrophotometer, we monitored the binding of ANS (100 µM) to HdeA or HdeB (7 µM) by measuring the increase in fluorescence intensity of ANS at 25°C upon its binding to protein. Samples were excited at 395 nm, with emission being recorded between 400 and 600 nm. Fluorescence readings were made for triplicate samples.

Oligomeric forms of HdeA and HdeB. The molecular weight of HdeA and HdeB was determined by filtration of the proteins on a TSK G-2000-SW HPLC gel permeation column (Hewlett Packard). Blue dextran (2 MDa), yellow dextran (20,000 Da), cytochrome C (12,500 Da) and vitamin B12 (1382 Da) were used as molecular weight standards. For experiments at pH 7.5, the column was equilibrated in 20 mM Tris, pH 7.5, 100 mM NaCl at 20°C, loaded with 20 µl of protein (2.4 mg/ml) and eluted at a flow rate of 0.5 ml/min. For experiments at pH 3 and pH 2, the column was equilibrated with 150 mM Na_2SO_4 adjusted to these pHs with sulfuric acid. Proteins were detected by their absorbance at 280 nm. When both proteins were loaded onto the column, they were detected by SDS-PAGE (on a 18% acrylamide gel).

Acid stress sensitivity of the *hdeA* and *hdeB* mutants, and of these mutants complemented by plasmids pBAD33-*hdeA*, pBAD33-*hdeB* and **pBAD33-*hdeAB***. Mutant and parental strains were grown in LB medium containing 0.05% arabinose for 24 hours at 37°C with aeration, in the presence of the required antibiotics. They were diluted to 10^6 bacteria per ml, and incubated for 90 min in acidified LB medium (pH 2 or pH 3) at 37°C under aeration (9). A control was done at pH 7. Survival patterns were determined by plating bacteria overnight on LB agar plates at pH 7.

Reagents. Restriction enzymes were from Invitrogen, and the plasmid extraction kit was from Qiagen. ADH (from rabbit muscle, 36 kDa) and GAPDH (from *Saccharomyces cerevisiaeae*, 36 kDa), were from Sigma, and OppA was purified as described in Richarme and Caldas (22). All other chemicals were from Sigma and were reagent grade.

RESULTS

Expression and purification of HdeA and HdeB. The BL21 (DE3) strains, transformed with the recombinant expression vector *pET-21a-hdeB* was induced for 3 hours with 1 mM IPTG, and its periplasmic extract was prepared by a modification (13) of the osmotic shock procedure of Nossal and Heppel (20). The HdeB overproducing strain accumulates a periplasmic protein migrating in SDS-PAGE with an apparent molecular weight of 10 kDa, which matches the expected HdeB molecular weight of 9 kDa (after processing of the signal sequence) **(Figure 1, lane 2).** The overexpressed protein represents 29 % in mass of the proteins in the induced periplasmic extract. HdeB was purified, as described in "Materials and Methods", by two chromatographic steps on a DEAE-Sephacel column and a hydroxyapatite column, **(Figure 1, lane 3),** and its identity was confirmed by mass spectrometry (not shown). We purified HdeA from strain *pET-21a-hdeA*, using the same osmotic shock procedure and chromatographic steps as for HdeB (**Figure 1, lane 4**), and confirmed its identity by mass spectrometry (not shown).

Acid-induced aggregation of periplasmic extracts from the *hdeA* and *hdeB* mutants. Periplasmic protein extracts are relatively resistant to acid-induced aggregation (18), and the acid-stress chaperone HdeA contributes to this resistance (9, 14). We investigated the acid-induced aggregation of periplasmic extracts from the *hdeA* and *hdeB* mutants and their parent, by analyzing pellets and supernatants after an acidic treatment at pH 2 or pH 3 for 60 min. The wild-type extract displays a moderate protein aggregation at acidic pH (**Figure 2A)**. In contrast, a massive protein aggregation is observed at both pHs in periplasmic extracts from the *hdeA* mutant (as reported previously (14)), and from the *hdeB* mutant. Conversely, a lower quantity of proteins from the two mutants (as compared to their parent) remains in the supernatant at these acidic pHs (**Figure 2B**). For both mutants, protein aggregation is more extensive at pH 2 than at pH 3, and for the *hdeA* periplasmic extract than for the *hdeB* extract. Furthermore, the proteins that appear to aggregate in periplasmic extracts from both mutants are similar. A fraction of HdeA (characterized by N-terminal sequencing) is found in the pellets at pH 2 (slightly above the 7 kDa marker in Figure 3A, except in the HdeA-deficient extract). At pH 7, there is no significant protein aggregation, suggesting that HdeA and HdeB do not function as neutral pH chaperones..

The two mutants display a strong protein aggregation at both pH 2 and pH 3, but HdeA and HdeB are mainly involved in periplasmic protein solubilization, at pH 2 and pH 3, respectively (see below). We checked the expression level of HdeA and HdeB in each mutant. Our 2D-gel electrophoresis analysis (performed as described in (17)) of periplasmic extracts from the *hdeA* and *hdeB* mutants showed that HdeA and HdeB are both undetectable in the *hdeA* mutant (which consequently behaves like a *hdeAB* mutant), and that HdeB is undetectable in the *hdeB* mutant, while HdeA is expressed at around 24% of the wild-type level (not shown), The null expression of the two chaperones in the *hdeA* mutant explains its aggregation phenotype at pH 2 and pH 3. The aggregation phenotype of the *hdeB* mutant cannot be explained by its low HdeA level, since complementation of the mutant by the P_{BAD} HdeA expression plasmid (the complemented strain expresses HdeA at a level that is 148% of that of the wild-type) does not significantly modify its aggregation behaviour (not shown).

HdeA is more efficient than HdeB in preventing periplasmic protein aggregation at pH 2. Periplasmic extracts from the parental strain and from the *hdeA* and *hdeB* mutants, either alone or supplemented with purified HdeA, HdeB, or both, were incubated at pH 2 for 60 min, and their 15,000 x g pellets and supernatants were analyzed. As reported above, protein aggregation at pH 2 increases in extracts from both mutants. The addition of HdeA decreases the aggregation of the *hdeA* (**Figure 3A)** and *hdeB* (not shown) extracts, whereas the addition of HdeB is much less efficient. HdeA and HdeB, together, are no more efficient than HdeA on its own. For the different periplasmic extracts, a high quantity of protein in the supernatant fraction (**Figure 3B**) corresponds to a low quantity in the pellet (**Figure 3A**), and vice versa. Most of HdeA and HdeB remains in the supernatant. We quantified the aggregated proteins in the *hdeB* mutant extract and in its wild-type control using NIH 1.62 software (**Figure 3C**). The profile of the HdeB-supplemented extract is only slightly different from that of the unsupplemented extract, whereas the profile of the HdeA-supplemented extract approaches

that of the parental strain extract. These results are consistent with the hypothesis that HdeA is the main chaperone involved in protein solubilization at pH 2. The important role of HdeA in preventing protein aggregation at pH 2 has already been discussed (9, 14).

HdeB is more efficient than HdeA in preventing periplasmic protein aggregation at pH 3. Periplasmic extracts from the parental strain and from the *hdeA* and *hdeB* mutants, either alone or supplemented with HdeA, HdeB, or both, were incubated at pH 3 for 60 min, and their 15,000 x g pellet and supernatant were analyzed. As reported above, there is a strong protein aggregation in extracts from both mutants. HdeA decreases the aggregation of the *hdeA* (**Figure 4A)** and *hdeB* (not shown) extracts, but HdeB is much more efficient than HdeA. HdeA and HdeB, together, are no more efficient than HdeB on its own. For the different periplasmic extracts, a high quantity of protein in the supernatant fraction **(Figure 4B)** corresponds to a low quantity in the pellet, and vice versa. We quantified the aggregated proteins in the *hdeA* mutant extract and in its wild-type control from Figure 4A using NIH 1.62 software (**Figure 4C**). The protein profiles of the HdeA- and HdeB-supplemented extracts reflect the greater efficiency of HdeB in solubilizing periplasmic proteins at pH 3. These results are consistent with the hypothesis that HdeB is the main chaperone for periplasmic protein solubilization at pH 3, and clarify the poor efficiency of the HdeA chaperone at pH 3 (14, this study). These results, together with those presented in the preceding paragraph, suggest that, to achieve the solubization of periplasmic extracts (the natural substrate of HdeA and HdeB) the task is divided between the two chaperones, HdeA being involved in protein solubilization mainly at pH 2, and HdeB mainly at pH 3.

Solubilization of model substrate proteins at pH 2 and pH 3. We examined the aggregation–suppressing function of HdeA and HdeB, using two usual substrates of chaperone activity assays, alcohol dehydrogenase (ADH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the periplasmic oligopeptide receptor OppA. Protein aggregation was monitored by analysis of 15,000 x g supernatants and pellets, after incubation of these proteins at acidic pHs in the absence or presence of chaperones. In the absence of chaperones, GAPDH remains in the supernatant at pH 7 and 4 (not shown), but not at pH 3 and 2 (**Figure 5A**). At pH 2, HdeA increases the fraction of soluble GAPDH from 12% to 81%, whereas HdeB has only a weak effect, and HdeA and HdeB together are no more efficient than HdeA on its own. At pH 3, HdeA and HdeB, separately, increase the fraction of soluble GAPDH from 1% to 12% and 8%, respectively, whereas HdeA and HdeB together solubilize up to 55% of GAPDH, suggesting that a synergy occurs between the two chaperones for the solubilization of this substrate protein to take place. For each experiment, the amounts of GAPDH found in the supernatant and the pellet are additive (in all the following experiments, the initial amount of each substrate protein added was quantitatively recovered in its supernatant and pellet fractions).
Experiments similar to that shown in Figure 6A were performed with ADH and OppA. For ADH solubilization, HdeA, but not HdeB, was efficient at pH 2 (98% solubilization); at pH 3, HdeA or HdeB, alone, were relatively inefficient (less than 10% solubilization) whereas HdeA and HdeB together solubilized up to 68% of the protein (**Figure 5B**). For OppA, HdeA solubilized 95% and 52% of the protein at pH 2 and pH 3, respectively, whereas HdeB solubilized 14% and 25% of OppA; the addition of both proteins at the same time was not significantly more efficient than HdeA alone (**Figure 5C**). These results are consistent with the hypothesis that HdeA makes a major contribution for the solubilization of model proteins at pH 2, that the efficiency of each chaperone is variable for protein solubilization at pH 3, and a synergy between the two chaperones occurs to enable the solubilization of the two cytoplasmic proteins tested at pH 3 (ADH and GAPDH) to take place.

HdeB exposes surfaces that are less hydrophobic than those of HdeA at acidic pHs. It is generally believed that molecular chaperones interact with their unfolded substrate proteins via hydrophobic interactions. HdeA exposes hydrophobic surfaces at acidic pH, but not at neutral pH, in accordance with the appearrance of its chaperone activity at acidic pHs (14). We measured the fluorescence emission of the hydrophobic probe ANS, in the presence of HdeA or HdeB, at different pHs. As shown in **Figure 6**, ANS binding to HdeA and HdeB is negligible at pH 8, and increases at pH 3 and pH 2 (ANS fluorescence is stronger at pH 2 than at pH 3, and stronger with HdeA than with HdeB). Furthermore, the blue shift of ANS fluorescence is similar for both chaperones at pH 2 (around 30

nm), but lower for HdeB (11 nm) than for HdeA (35 nm) at pH 3. This suggests that the surface of HdeB is less hydrophobic than that of HdeA, especially at pH 3. Such a difference in the exposed hydrophobicity of these two chaperones might explain their different chaperone properties described above, and the combination of both chaperones, under certain circumstances, might be required for the optimal handling of unfolded proteins.

Oligomeric forms of HdeB at neutral and acidic pHs. HdeB was analyzed by size-exclusion chromatography on a SW G-2000 HPLC column, as described in Materials and Methods. At pH 7.5, HdeB (loaded at 2.4 mg/ml) migrates as a dimer with an apparent molecular weight of around 16,000 Da (**Figure 7),** whereas at both pH 3 (**Figure 7**) and pH 2 (not shown), it migrates as a monomer (9,000 Da). At pH 7.5, HdeA (loaded at 2.4 mg/ml) migrates as a dimer (as previously reported (14)) with an apparent molecular weight of around 17,000 Da (not shown), whereas at pH 3 it migrates as a mixture of monomers and dimers (with an apparent molecular weight of around 14,000 Da) and at pH 2 as a monomer (10,000 Da) (not shown), as reported previously (14). Thus, like HdeA, HdeB dissociates from dimers to monomers at acidic pHs, but, whereas the dissociation of HdeB is complete at pH 3, that of HdeA is not. This difference might explain why HdeB is a more efficient chaperone than HdeA at pH 3.

We also checked whether HdeA and HdeB form a complex at pH 3. When a mixture of HdeA and HdeB (2.4 mg/ml, each) was loaded onto the SW G-2000 HPLC column equilibrated at pH 3, HdeA migrated (as it does when alone) as a mixture of monomers and dimers with an apparent molecular weight of around 14,000 Da,, and HdeB migrated (as it does when alone) as a monomer of around 9,000 Da (not shown).

Acid stress sensitivity of the *hdeA* and *hdeB* mutants and of these mutants complemented by pBAD33-*hdeA*, pBAD33-*hdeB* and pBAD33-*hdeAB*. Cultures of the mutants, and of their parent were incubated for 90 min at 37°C at either pH 2 or pH 3 (a control was done at pH 7), as described under "Materials and Methods", and their survival patterns were observed on LB plates at pH 7. The *hdeA* mutant, which contains neither HdeA nor HdeB (see above), displays 4% and 3% survival rates at pH 3 and pH 2, respectively. The HdeB mutant, which contains a reduced amount of HdeA (24% of the wild-type level (see above)) and no HdeB displays 8% and 7% survival rates at pH 3 and pH 2, respectively. (**Figure 8**). This suggests that, both genes are required for resistance of *E. coli* to these acidic pHs (the involvement of *hdeA* in *E. coli*'s resistance at pH 2 has already been reported (9)). However, since the *hdeA* mutant expresses neither HdeA nor HdeB, and since the *hdeB* mutant expresses 24% of the wild-type level of HdeA, in order to determine the respective contribution of each chaperone in acid-stress resistance, we complemented these mutants with pBAD vectors expressing HdeA or (and) HdeB under the control of the arabinose P_{BAD} promoter.

The viability of the *hdeA* mutant complemented with pBAD33-*hdeA* (this strain contains HdeA (140% of the wild-type level), but no HdeB) increased to 12% (a 3-fold increase) and 18% (a six-fold increase) after the pH 3 and pH 2 stresses, respectively. This suggests that HdeA alone affords some protection *in vivo* against acid stresses at these pHs.

The viability of the *hdeA* mutant complemented with pBAD33-*hdeB* (this strain contains HdeB (175% of the wild-type level) but no HdeA) increased to 16% (a 4-fold increase) and 11% (a 4-fold increase) after the pH 3 and pH 2 stresses, respectively. This suggests that HdeB alone affords a moderate protection of bacteria against acid stresses at these pHs.

The viability of the *hdeB* mutant complemented with pBAD33-*hdeA* (this strain contains HdeA (148% of the wild-type level) but no HdeB) increased to 9% and 16% after the pH 3 and pH 2 stresses, respectively. These viabilities are not very different (especially at pH 3) from those of the unsupplemented *hdeB* mutant (8% and 7%, respectively). Importantly, this suggests that the low viability of the uncomplemented *hdeB* mutant is not a consequence of its reduced HdeA level (25% of the level of the parental strain) but results indeed from an HdeB deficiency.

The viability of the *hdeB* mutant expressing the *hdeB* plasmid (this strain contains HdeA (28% of the wild-type level) and 125% of the wild-type HdeB level) increased to 28% and 18% after the pH 3 and pH 2 stresses, respectively.

The viabilities of the *hdeA* and *hdeB* mutants expressing the *hdeAB* plasmid attained 50-75% of the wild-type level, suggesting that the P_{BAD} expression system satisfyingly complements the strains deficient in acid-stress chaperones.

In short, these viability experiments suggest that both HdeA and HdeB are required for an optimal protection of bacteria against acid stress *in vivo*, at either pH 3 or pH 2 (especially since the *hdeA*- and the *hdeB*-deficient strains display a lower viability when complemented with a single chaperone than when complemented with both chaperones).

DISCUSSION

We have cloned, overexpressed, purified and characterized HdeB as a novel acid-stress chaperone. HdeB was found in the periplasm in a soluble form. It was purified by osmotic shock, followed by two chromatographic steps on DEAE-Sephacel and hydroxyapatite columns, and identified by MALDI-ToF mass spectrometry. We purified HdeA in parallel in order to compare the two chaperones.

A periplasmic extract from the *hdeB* mutant aggregates at pH 2 and pH 3, like an extract from the *hdeA* mutant (9). The aggregation defect of the hdeB mutant does not result from the lower expression (24% of the wild-type level) of HdeA since complementation of this mutant by the HdeA expression plasmid did not rescue its aggregation defect. Thus, HdeB is important for the solubility of the bacterial periplasm at acidic pH.

At pH 2, HdeA is the main chaperone involved in the *in vitro* solubilization of periplasmic extracts and of the model substrate proteins used in our study (the chaperone properties of HdeA at a pH around 2 have already been described by others (9, 14)). HdeB is much less efficient than HdeA in solubilizing periplasmic extracts at pH 2, and its best performance at this pH was the 25% solubilization of OppA. Furthermore, HdeB did not increase the efficiency of HdeA at pH 2.

At pH 3, HdeB solubilizes periplasmic extracts more efficiently than HdeA. It can prevent the aggregation of periplasmic extracts in the absence of HdeA, and thus functions as an acid-stress chaperone by itself. HdeA or HdeB, separately, are relatively inefficient in solubilizing GAPDH and ADH (less than 10% of these proteins were solubilized), and there is a functional synergy between them (leading to 52% and 68% solubilization of GAPDH and ADH, respectively). The occurrence of a synergy between HdeA and HdeB seems to depend on the particular substrate protein used (the synergy observed for the solubilization of the cytoplasmic proteins ADH and GAPDH is not obvious for that of OppA), or on chaperone / substrate ratios (at lower chaperone concentrations, we also observed some synergy between HdeA and HdeB for the solubilization of periplasmic extracts at pH 3, unpublished results).

Like HdeA, HdeB displays more hydrophobic surfaces at acidic pHs than at neutral pH, as judged from ANS fluorescence spectra. The surface of HdeB, however, appears less hydrophobic than that of HdeA, and such a difference might explain their respective roles in the renaturation of unfolded proteins.

Like HdeA, HdeB dissociates from dimers at neutral pH to monomers at acidic pHs. The dissociation of HdeB into monomers, however, is complete at pH 3 whereas that of HdeA is not. The easier dissociation of HdeB into monomers possibly explains its better chaperone properties at pH 3. Although HdeA and HdeB appear to cooperate in the solubilizaton of several protein substrates at pH 3, we could not detect the formation of a stable heterodimer between the two chaperones at this pH.

An *hdeB*-deficient strain, like an *hdeA*-deficient strain (9), displays an increased sensitivity to acid stress at pH 2 and pH 3 (and its sensitivity to acid stress is not a consequence of its reduced HdeA level), suggesting that HdeB, like HdeA, is involved in resistance to acid stress. *In vivo*, there seems to be a requirement for both chaperones for an optimal resistance of bacteria to acid stresses at either pH 3 or pH 2, in accordance with their co-expression from the same operon. This contrasts somewhat with results obtained *in vitro* suggesting that HdeA is more efficient at pH 2 and HdeB at pH 3. In fact, there are many differences between the *in vivo* and *in vitro* situations, including differences in protein concentrations and in the physico-chemical properties of the medium.

HdeA and HdeB are general chaperones (like DnaK, GroEL, small Hsps and periplasmic chaperones that also function as general chaperones (3, 6)), since they reduce the aggregation of many different periplasmic proteins. Furthermore, whereas HdeA and HdeB display a different pH specificity *in vitro* (pH 2 for HdeA, pH 3 for HdeB), they solubilize roughly the same proteins, since the profile of aggregated proteins at pH 2 in the presence of HdeA is similar to that of aggregated proteins at pH 3 in the presence of HdeB. Finally, the *hdeA* and *hdeB* mutants do not present a protein aggregation phenotype at neutral pH, suggesting that HdeA and HdeB are not involved in protein solubilization at neutral pH (this has previously been reported for HdeA (14)). Computational and structural

403 experiments will allow us to understand further the different specificities of these two chaperones
404 which have been designed by enterobacteria to prevent periplasmic protein aggregation at acidic pH.
405
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REFERENCES

1. Baba, T., Ara, T., Okumura, Y., Hasegawa, M., Takai, Y., Baba, M., Datsenko, K.A., Oshima, T., Tomita, M., Wanner, B.L., and H. Mori. 2006 Construction of *Escherichia coli* K-12 in frame, single-gene knock out mutants, the Keio collection . Mol. Systems Biol., doi : 10. 1038 / msb4100050.
2. Booth, I.R., P. Cash, and C. O'Byrne. 2002. Sensing and adapting to acid stress. Antonie Van Leeuwenhoek. **81**:33-42.
3. Bukau, B., and A.L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. Cell. **92**:351-366.
4. Datsenko K.A., and B.L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. **97**:6640-6644.
5. De Angelis, M., and M. Gobbetti. 2004. Environmental stress responses in *Lactobacillus*: a review. Proteomics **4**:106-122.
6. Duguay, A.R. and T.J. Silhavy. 2004. Quality control in the bacterial periplasm. Biochim. Biophys. Acta. **1694** : 121-134
7. Ferrero, R.L., V. Cussac, P. Courcoux, and A. Labigne. 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174**:4212-4217.
8. Foster, J.W. 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat. Rev. Microbiol. **2**:898-907.
9. Gajiwala, K.S, and S.K. Burley. 2000. HdeA, a periplasmic protein that supports acid resistance in pathogenic enteric bacteria.. J. Mol. Biol. **295**:605-12.
10. Goto, Y., and A.L. Fink. 1989. Conformational states of beta-lactamase: molten-globule states at acidic and alkaline pH with high salt. Biochemistry. **28**:945-952.
11. Guzman, L.M., Belin, D., Carson, M.J., and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177** :4121-4130.
12. Hersh, B.M., F.T. Farooq, D.N. Barstad, D.L. Blankenhorn, and J.L. Slonczewski. 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. J. Bacteriol. **178**:3978-3981.
13. Higgins, C.F., and M.M. Hardie. 1983. Periplasmic protein associated with the oligopeptide permeases of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **155**:1434-1438.
14. Hong , W., W. Jiao, J. Hu, J. Zhang, C. Liu, X. Fu, D . Shen, B. Xia, and Z. Chang. 2005. Periplasmic protein HdeA exhibits chaperone-like activity exclusively within stomach pH range by transforming into disordered conformation. J Biol Chem. **280**:27029-27034
15. Koebnik, R., K.P. Locher, and P. Van Gelder. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. Mol. Microbiol. **37**:239-53.
16. Laemmli, U..K. 1972. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680-685
17. Lim, E.M., S.D. Ehrlich, and E. Maguin. 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis. **21**:2557-2561.
18. Liu Y, X. Fu X, J. Shen, H. Zhang, W. Hong, and Z. Chang. 2004. Periplasmic proteins of *Escherichia coli* are highly resistant to aggregation: reappraisal for roles of molecular chaperones in periplasm. Biochem. Biophys Res. Commun. **316** : 795-801.
19. Miller, J.H. 1972. Experiments in molecular genetics, p 421. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.
20. Nossal, N.G., and L.A. Heppel. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J Biol Chem. **241**:3055-3062.
21. Richard, H., and J.W. Foster. 2004. *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J. Bacteriol. **186**:6032-6041
22. Richarme, G. and T.D. Caldas. 1997. Chaperone properties of the bacterial periplasmic substrate-binding proteins. J. Biol. Chem. **272**:15607-15612.
23. Schirmer, T. 1998. General and specific porins from bacterial outer membranes. J Struct Biol. **121**:101-109.
24. Small, P.L., and S.R. Waterman. 1998. Acid stress, anaerobiosis and gadCB: lessons from *Lactococcus lactis* and *Escherichia coli*. Trends Microbiol. **6**:214-216.

25. Thompson, S.A., and M.J. Blaser. 1995. Isolation of the *Helicobacter pylori* recA gene and involvement of the recA region in resistance to low pH. Infect. Immun. **63**:2185-2193.
26. Tucker, D.L., N. Tucker, and T. Conway. 2002. Gene expression profiling of the pH response in *Escherichia coli*. J. Bacteriol. **184**:6551-6558.
27. Yohannes, E., D.M. Barnhart, and J.L. Slonczewski. 2004. pH-dependent catabolic protein expression during anaerobic growth of *Escherichia coli* K-12. J. Bacteriol. **186**:192-199.

LEGENDS TO FIGURES

Figure 1. Purification of HdeA and HdeB. Protein samples were separated by dodecylsulfate polyacrylamide gels (16%), and stained with Coomassie brillant blue. *Lane 1*, periplasmic extract from uninduced strain BL21(DE3), *pET-21a-hdeB*; *lane 2*, periplasmic extract from BL21(DE3), *pET-21a - hdeB* induced for 3 hours with IPTG; *lane3*, 5 µg of purified HdeB; *lane 4*, 5 µg of purified HdeA. The positions of molecular weight markers are indicated on the left.

Figure 2. Acid-induced aggregation of periplasmic extracts. SDS-PAGE analysis of the pellet (A) and supernatant (B) fractions of periplasmic extracts (20 µg each) from the *hdeA* mutant, *hdeB* mutant and their parent, after an acid treatment at pH 2 or at pH 3 for 60 min (a control was made at pH 7).

Figure 3. Prevention of periplasmic protein aggregation at pH 2. SDS-PAGE analysis of the pellet (A) and supernatant (B) fractions of periplasmic extracts (20 µg) from the *hdeA* mutant and its parental strain, after incubation for 60 min at pH 2, either alone or in the presence of purified (15 µg each) HdeA, HdeB or both (7.5 µg each), C) Densitometric scan of the protein distribution of periplasmic extracts from the wild-type strain, and the *hdeB* mutant supplemented with HdeA or HdeB, as indicated. We checked that the intensities of protein bands (excluding the HdeA/B bands) in pellets and supernatants add up to similar amounts in all cases.

Figure 4. Prevention of periplasmic protein aggregation at pH 3. A) SDS-PAGE analysis of pellet (A) and supernatant (B) fractions of periplasmic extracts (20 µg) from the *hdeA* mutant and its parental strain, after incubation for 60 min at pH 3, either alone or in the presence of purified (15 µg each) HdeA, HdeB or both (7.5 µg each), B) Densitometric scan of protein distribution of periplasmic extracts from the wild-type strain and the *hdeA* mutant supplemented with HdeA or HdeB, as indicated. We checked that the intensities of protein bands (excluding the HdeA/B bands) in pellets and supernatants add up to similar amounts in all cases.

Figure 5. Solubilization of GAPDH, ADH and OppA at acidic pH. A) SDS-PAGE analysis of the supernatant and pellet fractions of GAPDH (10 µg) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5 µg each) HdeA, HdeB, or both. B) SDS-PAGE analysis of the supernatant fractions of ADH (10 µg) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5 µg each) HdeA, HdeB, or both. C) SDS-PAGE analysis of the supernatant fractions of OppA (10 µg) that was subjected to treatment for 60 min at pH 3 or 2, either alone or in the presence of (5 µg each) HdeA, HdeB, or both. The molecular weights (monomeric form) of HdeA, HdeB, GAPDH, ADH and OppA are 10 kDa, 9 kDa, 36 kDa, 36 kDa and 61 kDa, respectively.

Figure 6. ANS fluorescence of HdeB and HdeA at neutral and acidic pHs. The intensity of ANS fluorescence (100 µM, in H_2SO_4 solution of pH 2 or pH 3, or in 10 mM Tris pH 8) in the presence of 7 µM HdeA or 7 µM HdeB was measured after excitation at 395 nm.

Figure 7. Oligomeric forms of HdeB at neutral and acidic pHs. For experiments at pH 7.5, the column was equilibrated in 20 mM Tris, pH 7.5, 100 mM NaCl at 20°C, loaded with 20 µl of HdeB (2.4 mg/ml) and eluted at a flow rate of 0.5 ml/min. For experiments at pH 3 and pH 2, the column was equilibrated with 150 mM Na_2SO_4 adjusted to these pHs with sulfuric acid, and loaded with 20 µl of HdeB or (and) HdeA (2.4 mg/ml each), equilibrated at the pH of the column). Proteins were detected by their absorbance at 280 nm. Blue dextran (2 MDa), yellow dextran (20,000 Da), cytochrome C (12,500 Da) and vitamin B12 (1382 Da) were used as molecular weight standards.

Figure 8. Acid stress sensitivity of the *hdeA* and *hdeB* mutants, and of these mutants complemented with plasmids pBAD33-*hdeA*, pBAD33-*hdeB* and pBAD33-*hdeAB*. The mutants, either uncomplemented (0) or complemented with pBAD33-*hdeA* (A), pBAD33-*hdeB* (B) or pBAD33-*hdeAB* (AB) and their parent were subjected to acid stress in LB medium adjusted to pH 3

(light grey) or pH 2 (dark grey) for 90 min at 37°C, as described in “Materials and Methods”, and survival patterns were determined by plating bacteria overnight on LB agar plates at pH 7. The results are the average ± SEM of three experiments. The colony counts of the mutants were normalized to those of the parental strain. All the strains displayed similar viabilities at pH 7.

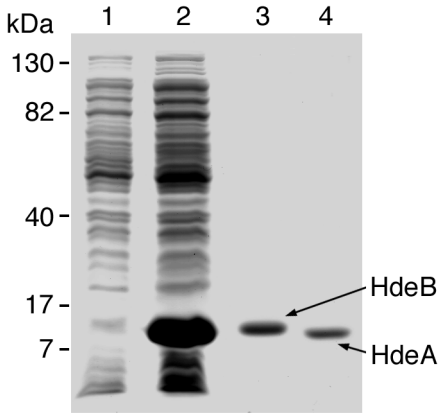

Figure 1

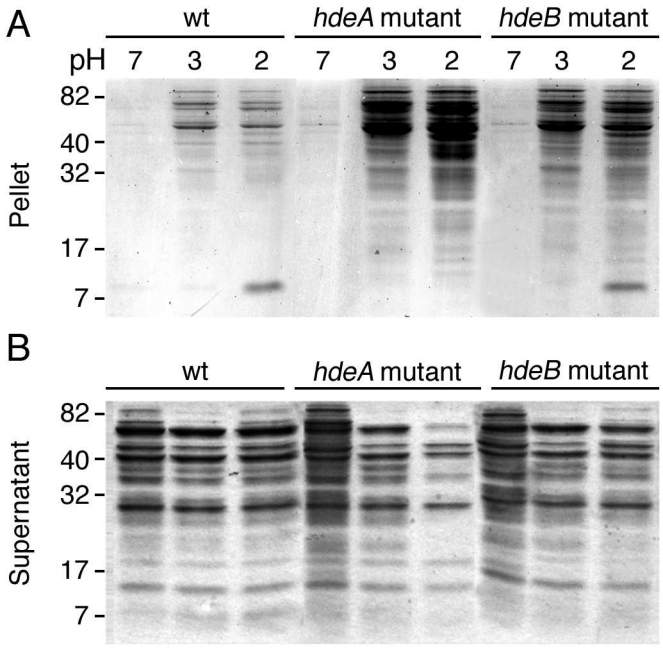
A
wt
hdeA mutant
hdeB mutant
pH 7 3 2 7 3 2 7 3 2
82
40
32
17
7
Pellet
B
wt
hdeA mutant
hdeB mutant
82
40
32
17
7
Supernatant

Figure 2

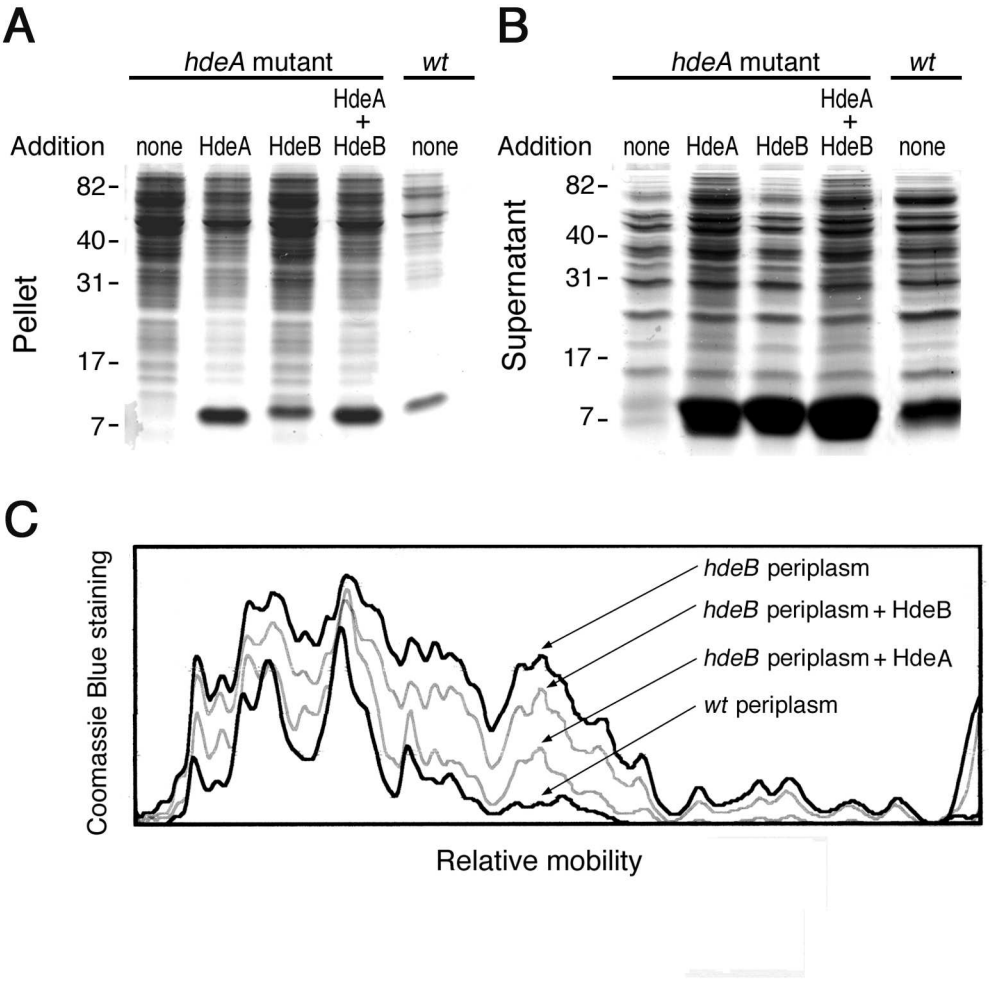
A
hdeA mutant
wt
HdeA + HdeB
Addition
none
HdeA
HdeB
HdeA + HdeB
none
82-
40-
31-
17-
7-
Pellet
B
hdeA mutant
wt
HdeA + HdeB
Addition
none
HdeA
HdeB
HdeA + HdeB
none
82-
40-
31-
17-
7-
Supernatant
C
Coomassie Blue staining
hdeB periplasm
hdeB periplasm + HdeB
hdeB periplasm + HdeA
wt periplasm
Relative mobility

Figure 3

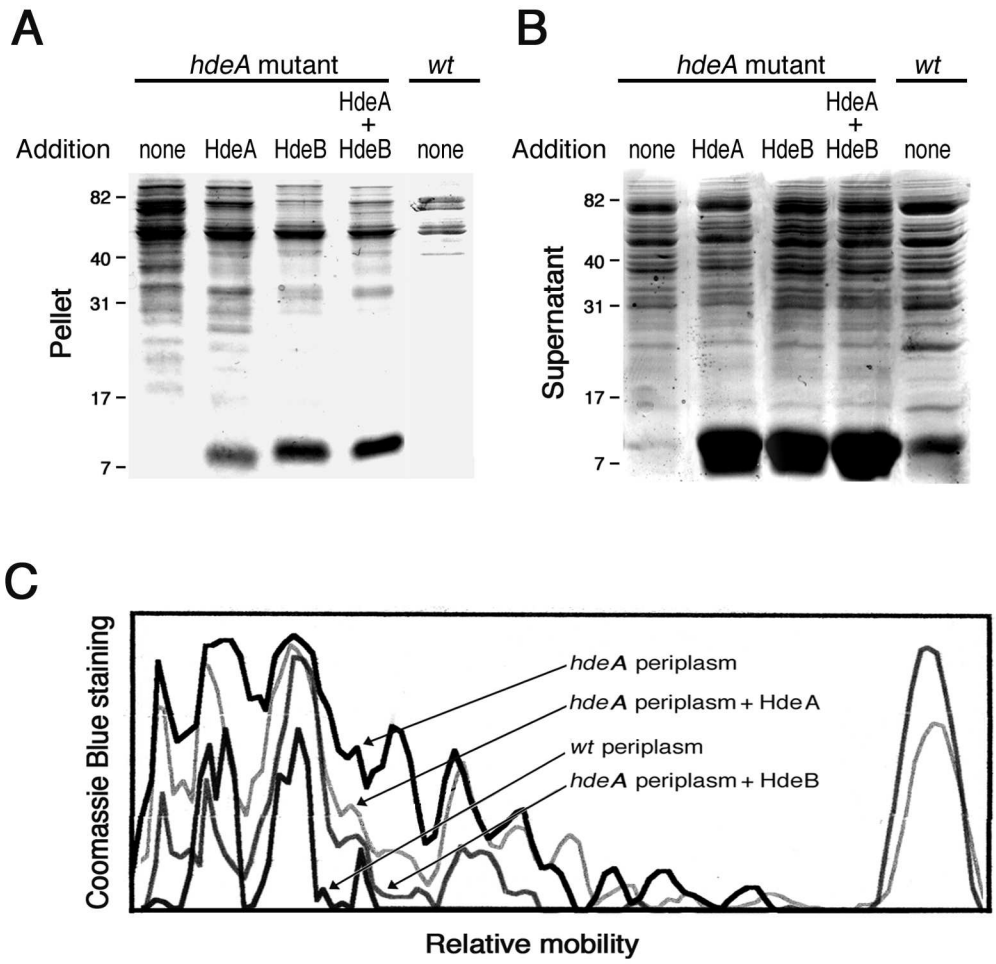
A
hdeA mutant
wt
Addition
none
HdeA
HdeB
HdeA + HdeB
none
82
40
31
17
7
Pellet
B
hdeA mutant
wt
Addition
none
HdeA
HdeB
HdeA + HdeB
none
82
40
31
17
7
Supernatant
C
Coomassie Blue staining
hdeA periplasm
hdeA periplasm + HdeA
wt periplasm
hdeA periplasm + HdeB
Relative mobility

Figure 4

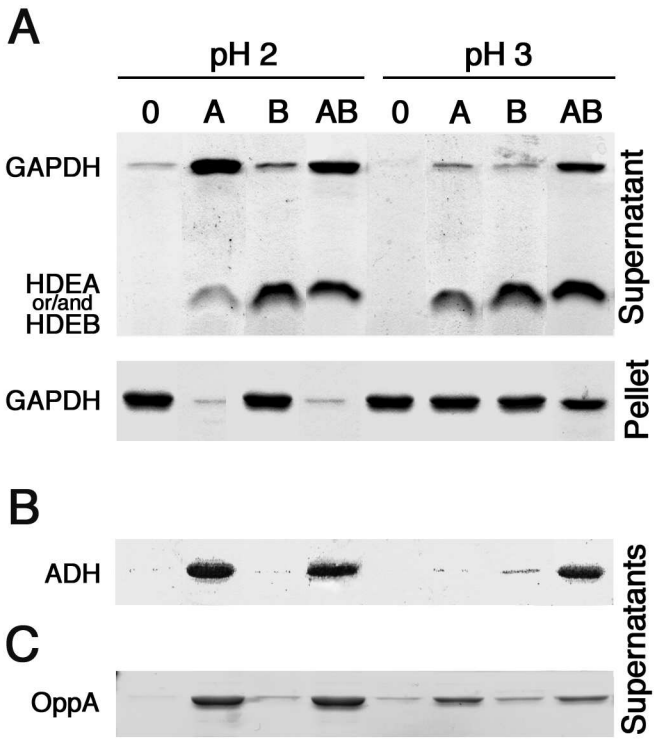
A
pH 2
pH 3
0
A
B
AB
0
A
B
AB
GAPDH
HDEA
or/and
HDEB
Supernatant
GAPDH
Pellet
B
ADH
Supernatants
C
OppA

Figure 5

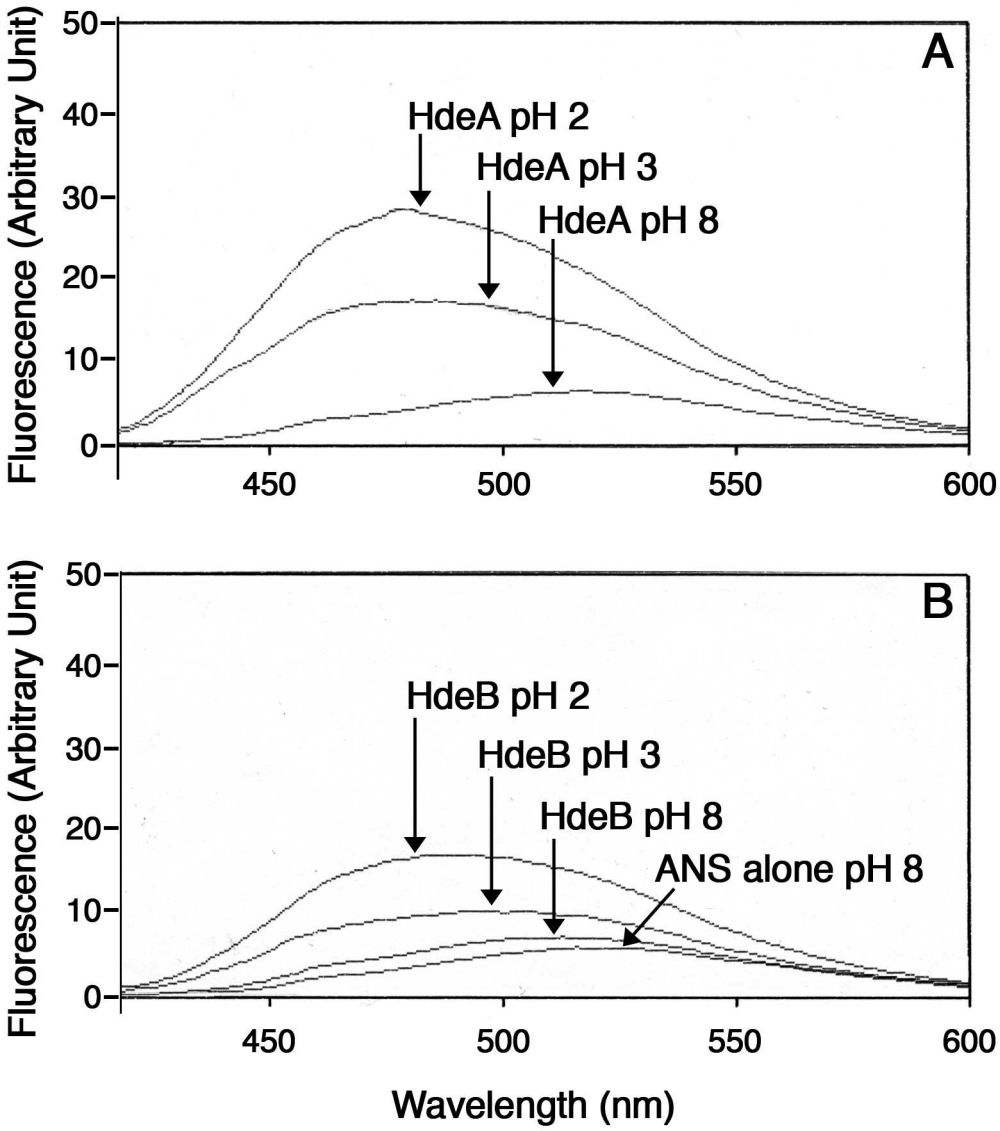
A
HdeA pH 2
HdeA pH 3
HdeA pH 8
Fluorescence (Arbitrary Unit)
50
40
30
20
10
0
450
500
550
600
B
HdeB pH 2
HdeB pH 3
HdeB pH 8
ANS alone pH 8
Fluorescence (Arbitrary Unit)
Wavelength (nm)

Figure 6

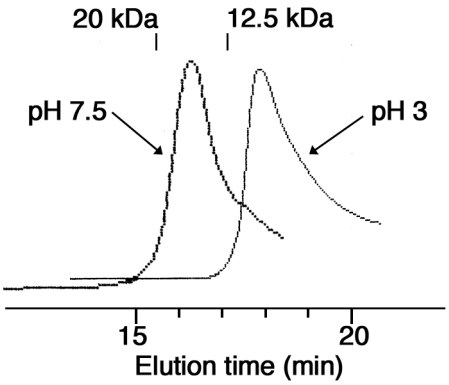

Figure 7

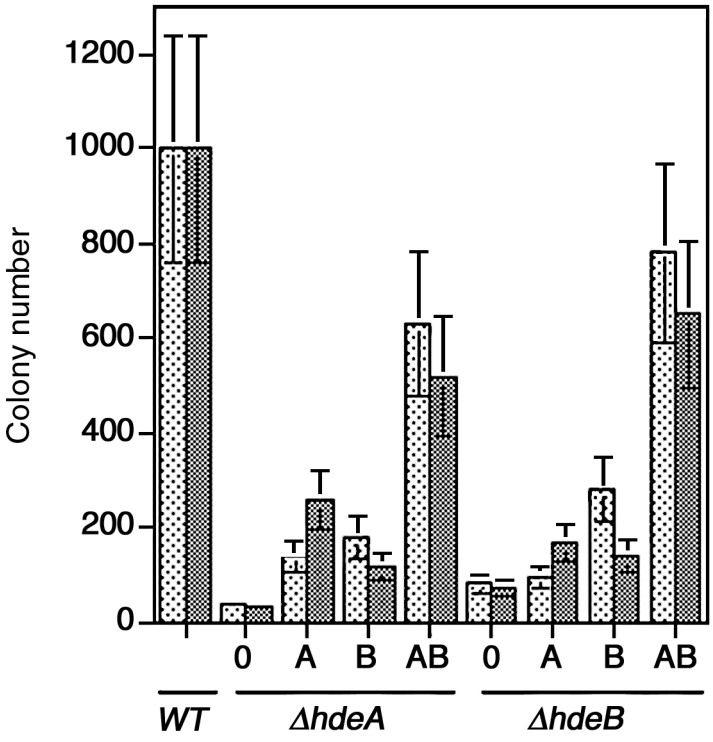
Colony number
1200
1000
800
600
400
200
0
0
A
B
AB
0
A
B
AB
WT
ΔhdeA
ΔhdeB

Figure 8